

ADMINISTRATION OF METHYL FARNESOATE THROUGH THE ARTEMIA VECTOR, AND ITS EFFECT ON *MACROBRACHIUM ROSENBERGII* LARVAE

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(Received 22.2.98, Accepted 19.5.98)

Abstract

We have developed a method for the administration of juvenile hormone-like compounds into crustacean larvae through a live food vector, the brine shrimp *Artemia*, commonly used in prawn aquaculture. In crustaceans, the only juvenile hormone-like compound found to date is methyl farnesoate (MF), the unepoxidated form of insect juvenile hormone III. Since MF is hydrophobic, its administration to crustacean larvae in aqueous culture media is problematic. Accumulation of the compound in *Artemia* cultured in a lipid medium enriched with MF was verified by HPLC, which demonstrated the stability of the compound within the vector. *Artemia* that were cultured in media containing [³H]MF accumulated 7.27% of the total radioactivity added. About 0.065% of the total radioactivity added was found in 20 *M. rosenbergii* larvae fed on the enriched *Artemia*. MF freshly administered daily to *M. rosenbergii* larvae caused a retardation of larval growth, manifested by carapace length. In addition, MF altered larval development by retarding the stage specific morphological features between larval stages 5 and 9. This new method for administering MF may facilitate further studies examining the regulatory role of MF in crustacean larval development and metamorphosis. It may also be instrumental in the administration of other hydrophobic drugs into crustacean larvae.

Introduction

Juvenile hormones—a family of epoxidated sesquiterpenoids secreted by the corpus allatum—act as growth regulators in insects (Wigglesworth, 1970). The first report of the presence of such a compound in Crustacea

was that of Laufer et al. (1987) who discovered methyl farnesoate (MF), the unepoxidated form of juvenile hormone III, in the spider crab *Libinia emarginata*. Since then, MF has been found in many crustaceans, including crabs,

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lobsters, crayfish (Borst et al., 1987; Laufer et al., 1987; Laufer and Borst, 1988; Landau et al., 1989), and the freshwater prawn *Macrobrachium rosenbergii* (Sagi et al., 1991).

In studies of the role of juvenoids such as MF in the larval development of crustaceans, the nature of the compound presents a problem in terms of the means of administration: MF is a hydrophobic compound with a solubility of about 10 μ M in aqueous solutions (Hammock and Mumby, 1978; Tobe et al., 1989) and a half life of about 30 min when injected into crustacean hemolymph (Takac et al., 1997). Since adding the juvenoid to the aqueous rearing media for larvae (Hertz and Chang, 1986; Borst et al., 1987; Ahl and Brown, 1990) or injecting it into larvae (Charmantier et al., 1988) is thus problematic, a new method of administration of juvenoids into crustacean larvae is needed.

We therefore attempted for the first time in crustaceans a method of administering a juvenoid via the food chain. Live food organisms, such as the brine shrimp *Artemia*, are extensively used as the main food sources in the larviculture of marine fish, shrimps and prawns. In aquaculture, *M. rosenbergii* larvae are reared on *Artemia* nauplii, either solely or in combination with prepared diets (New, 1990). Nutritional inadequacies of the brine shrimp have resulted in the development of enrichment techniques using fatty acid emulsions to increase the concentration of highly unsaturated fatty acids in the predator diet (Watanabe et al., 1983; Leger et al., 1987). In addition to nutritional enrichment, various antibiotics (Mohney et al., 1990; Verpraet et al., 1992; Aguilar-Aguila et al., 1994; Duis et al., 1995; Touraki et al., 1995; Touraki et al., 1996), vaccinations (Joosten et al., 1995) and hydrophobic steroids (Martin-Robichaud et al., 1994) have been incorporated into the brine shrimp.

Larval development and metamorphosis in some crustaceans are similar to these processes in hemimetabolous insects and involves the gradual manifestation of morphological changes until the first juvenile stage is reached (Snyder and Chang, 1986). *M. rosenbergii* represents a "common type" larval development (Sollaud, 1923), which includes 11 distinct stages followed by metamorphosis (Uno

and Soo, 1969). Each stage is significantly larger than the previous stage, and is characterized by distinctive carapace and telson spination, development of teeth on the rostrum, structural changes and setation in the pleopods and uropods, and structural changes in the pereopods, eyes and abdominal somites (Abdu, 1996).

In this study we derived and tested a new method for the administration of MF into larvae of *M. rosenbergii* via the *Artemia* vector. The effect of MF administered via this method on larval development and growth in *M. rosenbergii* was studied.

Materials and Methods

Enrichment of *Artemia* with MF. *Artemia* cysts (Neptune, Salt Lake City) were decapsulated according to the method described by Sorgeloos et al. (1986). The decapsulated cysts (4 g/liter) were hatched for 24 h at $28 \pm 1^\circ\text{C}$ in 20 g of technical grade sea salt per liter of distilled water, under continuous aeration and illumination (1,000 lux). After 24 h, instar I larvae were harvested, separated from hatching debris, and rinsed thoroughly. The hormones MF (methyl 3,7,11-trimethyl-2,6,10-dodecatrienoate) and [^3H]MF (sp. act. 11.5 Ci/mmol) were kindly supplied by Prof. Hans Laufer, University of Connecticut. Enrichment solutions were prepared by adding the hormones, dissolved in ethyl alcohol, to 0.2 g/liter of an oil (Super-Selco, *Artemia* System S.A., Baasrode, Belgium) emulsified in water. *Artemia* were exposed to the enrichment solution for 24 h at $28 \pm 1^\circ\text{C}$ in 20 g of technical grade sea salt per liter of distilled water under continuous illumination.

Qualitative analysis of MF from enriched *Artemia*. Nauplii, 3 g/500 ml (approximately 750 nauplii/ml), were enriched as described above in 200-ml glass bottles under continuous aeration. The enrichment solution contained 0.5 μ g MF/ml of Super-Selco in a ratio of 22% of the non-biological isomer, *cis-trans* MF, to 78% of the biological isomer *all-trans* MF. After enrichment, the *Artemia* nauplii were rinsed thoroughly and homogenized in 2 ml of 4% NaCl and 5 ml of acetonitrile. The *Artemia* homogenates were filtered through α -cellu-

lose, washed twice with 40 ml of 4% NaCl and 30 ml of acetonitrile, and extracted twice with 30 ml of hexane each time. The hexane fraction was evaporated off by means of a rotary evaporator. The dry matter was dissolved in 7 ml of hexane and passed through a Sep-Pak silica cartridge (Waters, Milford, MA), and the cartridge was rinsed with methylene chloride. The methylene chloride solution was evaporated, redissolved with 100 μ l of hexane and analyzed by HPLC according to Sagi et al. (1991).

Administration of MF via the *Artemia* vector to *M. rosenbergii* larvae. *Artemia* nauplii, 4,000/8 ml, were enriched as described above with agitation in a 55-mm diameter glass Petri dish. Each enrichment solution contained a negligible fixed amount of [3 H]MF (8×10^4 dpm/ml of the culture medium) mixed with 0.2 μ g/ml non-radioactive MF in Super-Selco. This MF dose was chosen since up to 0.2 μ g MF/ml a highly significant correlation was found between accumulation of MF in *Artemia* and the concentration of MF in the enrichment medium ($p < 0.001$; $r = 0.993$). Moreover, Ahl and Brown (1990) showed that doses above 0.25 μ g/ml may be toxic to *Artemia*. To measure the level of MF in the *Artemia* at the end of the 24-h enrichment period, 100 *Artemia* were sampled and homogenized in 0.05M Tris buffer, pH 7.4, and radioactivity was counted in a scintillation counter (Packard, Groningen). The rest of the *Artemia* was given as food to 20 *M. rosenbergii* larvae reared in 60 ml of seawater that had been sterilized and diluted to 13 ppt with distilled water. After 8 h, the larvae were collected and homogenized, and radioactivity was counted. The radioactivity reflected the concentration of accumulated MF, which was expressed in ng MF per 4,000 *Artemia* nauplii and ng MF per 20 *M. rosenbergii* larvae.

Bioassay for MF activity. *M. rosenbergii* larvae were hatched and reared according to Daniels et al. (1992). In the experiment, the larvae were treated with three doses of MF by feeding them *Artemia* raised in 0.02, 0.11 or 0.20 μ g MF/ml. The control group was fed with *Artemia* enriched with the MF vehicle alone (ethyl alcohol and Super-Selco). Each treatment was administered in three replicates, each containing 15 *M. rosenbergii* larvae at

stage IV, determined according to Uno and Soo (1969). The experimental system consisted of 12 dark plastic containers (300 ml) that were constantly aerated. The water was changed daily, and the temperature was kept constant at $27 \pm 1^\circ\text{C}$. Larvae were fed daily *ad libitum* with *Artemia* nauplii freshly enriched with MF as described above. Five larvae per replicate were randomly sampled twice a week; their larval stage was determined (Uno and Soo, 1969), and their carapace length was measured with an ocular micrometer prior to being returned to the growth chamber. The average larval stage and the average carapace length were compared between treatments, and the statistical significance was tested using the Mann-Whitney U test.

Results

HPLC analysis of *Artemia* extracts indicated that MF from the enrichment medium accumulated in the *Artemia* (Fig. 1). The retention times of the peaks representing MF isomer standards, cis-trans and all-trans MF, were 4.00 and 4.87 min, respectively (Fig. 1a). Comparison of the peak areas of the two MF isomers identified in the HPLC separation of the MF enriched *Artemia* (Fig. 1b) showed a ratio of 22% cis-trans to 78% all-trans, which was the exact ratio of the MF mixture inserted into the enrichment media. No such MF peaks were found in the HPLC separation of non-enriched *Artemia* (Fig. 1c).

After 24 h of enrichment of *Artemia* with 1600 ng/8ml containing 4,000 *Artemia* (0.2 μ g MF/ml), 116.3 ± 25.6 ng of MF were found in 4,000 *Artemia* (Table 1). This value represents approximately 7.27% of the total MF added into the enrichment medium. When MF was administered via the *Artemia* vector to the target species, *M. rosenbergii* larvae, approximately 0.065% (1.04 ± 0.5 ng MF) of the original MF added to the *Artemia* enrichment medium was found in 20 *M. rosenbergii* larvae fed for 8 h on the enriched *Artemia*.

MF administered to *M. rosenbergii* via *Artemia* caused retardation of larval development, as manifested by larval stage (Fig. 2) while feeding and survival was similar in the control and all the treatments. The control

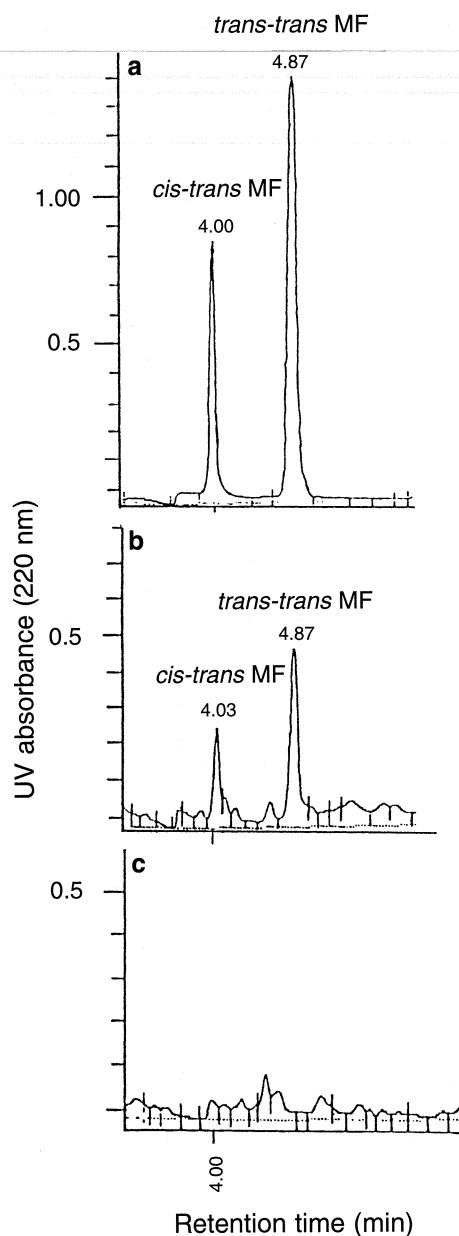


Fig. 1. HPLC analysis of MF and *Artemia* extracts: (a) chromatograph of MF isomers, 22% of the non-biological isomer, cis-trans MF, and 78% of the biological isomer, all-trans MF; (b) chromatograph of MF extracted from *Artemia* enriched with the above-described MF mixture; (c) chromatograph of an extract of non-enriched *Artemia*.

group developed rapidly, reaching an average stage of 8.3 after 8 days and an average stage of 9.6 at the end of the experiment (after 14 days). At the lowest dose of 0.02 μg MF/ml, the average stage of the larvae was significantly lower ($p < 0.001$) than that of the control group on day 14, with very slight larval development being recorded in the treatment group from day 8. Larvae fed on *Artemia* that were enriched with higher doses of MF (0.11 and 0.20 μg MF/ml) also manifested significantly slower development ($p < 0.001$) than the control group, starting from day 8. Larvae fed on *Artemia* enriched with 0.11 μg FM/ml developed significantly ($p < 0.001$) from day 8 to day 11, but this was followed by a significant retardation of larval development from day 11. Larvae fed *Artemia* enriched with 0.20 μg MF/ml ceased to develop after day 8. An additional effect of MF administered via *Artemia* on the growth of the larvae was manifested by carapace length (Fig. 3). The control group grew fastest; after 8 days the average carapace length was over 1 mm, and after 14 days the average carapace length was 1.2 mm. The larvae fed on *Artemia* enriched with 0.02 μg MF/ml showed significantly slower growth than the control group starting from day 8 ($p < 0.001$), and only very minute larval growth was recorded from that day onwards. Larvae fed on *Artemia* enriched with higher doses of MF (0.11 and 0.20 μg MF/ml) grew significantly slower than the control group, starting from day 4 ($p < 0.001$), and only very minute growth was recorded after that day.

Discussion

A new method was established in which, for the first time in Crustacea, a live vector was used for the administration of a hormone. This method is based on continuous exposure of *M. rosenbergii* larvae to MF, using *Artemia* that were freshly enriched every day as the vector. This method facilitates long-term exposure of the target species to known levels of MF (approximately 0.065% of the total MF added into the vector's enrichment medium). It presents an improvement over previously used administration methods (Hertz and Chang, 1986; Borst et al., 1987), in which juvenile hor-

Table 1. The accumulation of MF in the *Artemia* vector and its concentration in *Macrobrachium rosenbergii* larvae fed the enriched *Artemia*.

MF concentration in the enrichment medium (ng/8 ml) ¹	MF level in <i>Artemia</i> (ng/4,000 <i>Artemia</i>) ²	MF level in <i>M. rosenbergii</i> larvae (ng/20 larvae) ³
1600 (0.2 µg/ml)	116.3±25.6	1.04±0.5
100%	7.27±1.5%	0.065±0.02%

1 *Artemia* nauplii, 4,000/8 ml, were enriched with a negligible fixed amount of [³H]MF (8 x 10⁴ dpm/ml of the culture medium) mixed with 0.2 µg/ml non-radioactive MF in Super-Selco.

2 MF level in the *Artemia* was measured at the end of the 24-h enrichment period.

3 The 4,000 enriched *Artemia* were given as food to 20 *M. rosenbergii* larvae for 8 h.

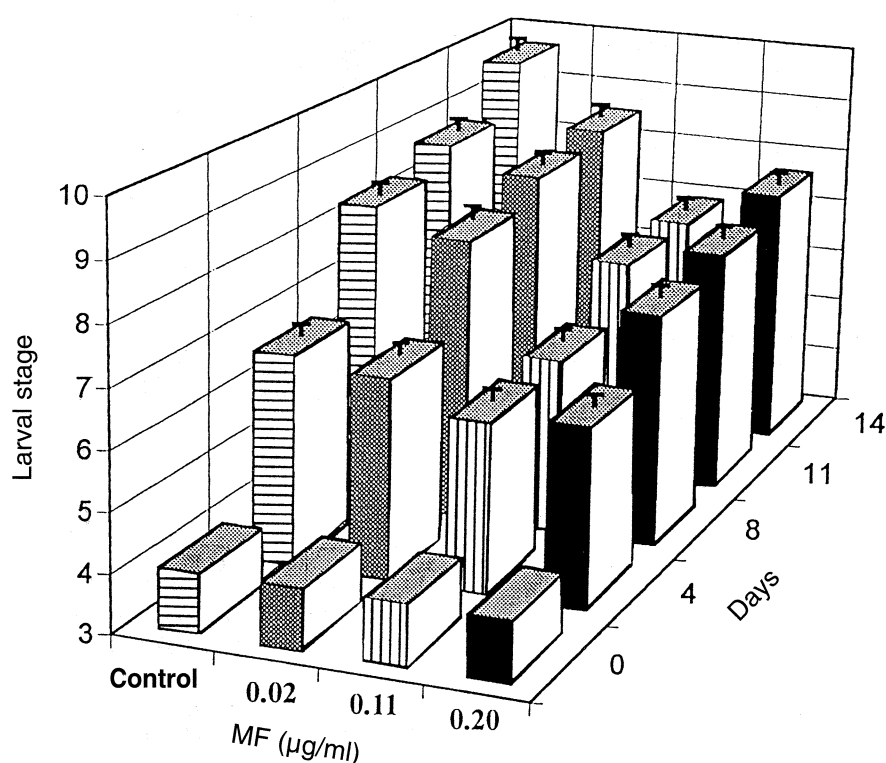


Fig. 2. Average larval stage of *Macrobrachium rosenbergii* during 14 days of feeding on *Artemia* enriched with different concentrations of MF. Concentration represents amount of MF added to the medium in which *Artemia* were incubated prior to being provided as food to the treated *M. rosenbergii* larvae. Each bar represents mean ± SE (n = 15).

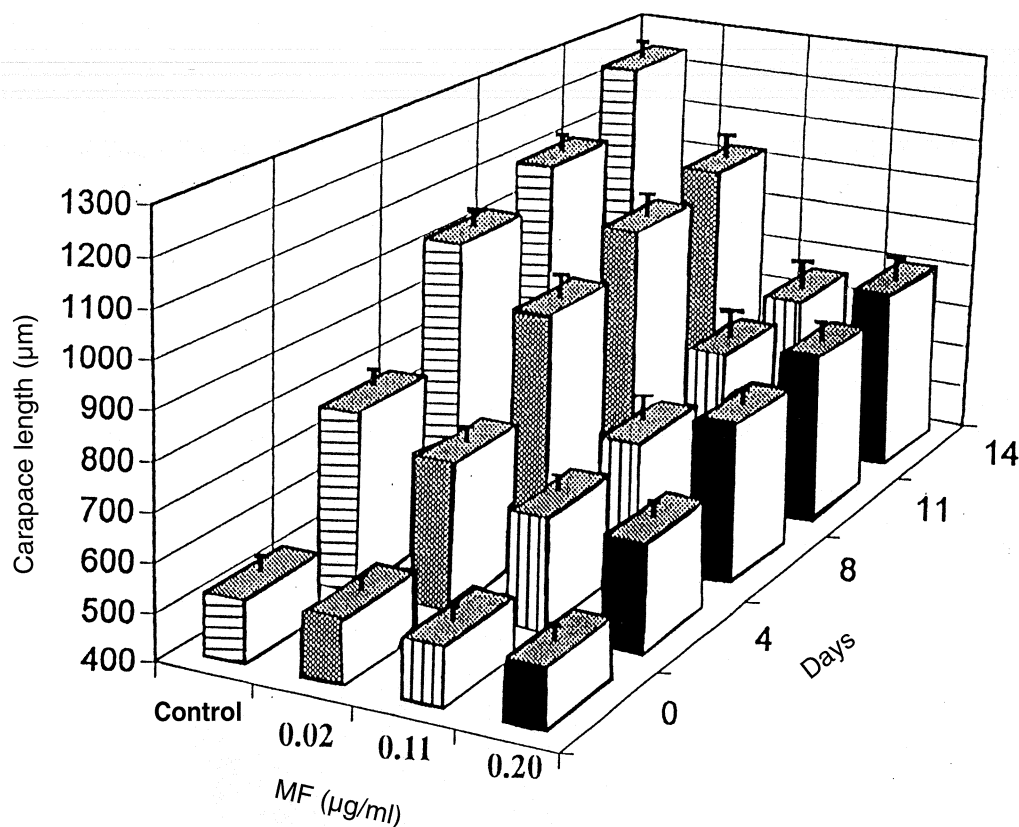


Fig. 3. Average carapace length of *Macrobrachium rosenbergii* larvae during 14 days of feeding on *Artemia* enriched with different concentrations of MF. Concentration represents amount of MF added to the medium in which *Artemia* were incubated prior to being provided as food to the treated *M. rosenbergii* larvae. Each bar represents mean \pm SE ($n = 15$).

mone III and MF were added to the rearing medium of lobster larvae (*Homarus americanus*), with no direct estimation of the actual amount of hormone reaching the target species. The latter methods dictated the use of concentrations which were several orders of magnitude (10^3) higher than the level of MF found in the hemolymph of lobster larvae (Borst et al., 1987). Using the injection method, the exposure period is limited due to the relatively short half life of MF in the crustacean body (Takac et al., 1997). Thus, prolonged exposure

using a live vector presents an improvement in juvenoid administration compared to the previous use of a single injection of juvenile hormone I to larvae (Charmantier et al., 1988), and is in accordance with the administration of MF through dry food to adult crustaceans (Laufer, pers. comm.).

Using the *Artemia* vector, approximately 0.065% of the MF added to the *Artemia* enrichment medium was found in 20 *M. rosenbergii* larvae feeding for 8 h on the enriched *Artemia*. This suggests that the level of MF adminis-

tered per larva in the present study did not exceed 0.065 ng MF for larvae exposed to the highest dose and 0.006 ng MF for larvae exposed to the lowest dose. Since the average weight of a larva is about 0.002 g, these levels could be expressed as 3-32 ng MF/g. The calculated level for the MF exposure is thus within the range of physiological level found in *M. rosenbergii* adults (up to 40 ng/ml; Sagi et al., 1991).

M. rosenbergii growth and development were retarded in larvae that were fed daily with MF administered through freshly enriched *Artemia*. This finding is the first instance in which a dose dependent effect of MF has been reported as a result of exposure of decapod larvae to non-pharmacological levels of MF. Comparable results have been reported in several insect species in which treatment with juvenile hormone inhibited larval growth and ecdysis and no, or very limited, morphogenesis was recorded (Lohri-Kaelin and Masner, 1981). In crustaceans, MF (Borst et al., 1987) and juvenile hormone III (Hertz and Chang, 1986) retarded larval development of *H. americanus*, and juvenile hormone analogues, such as methoprene and hydroprene, caused retardation of larval development of the mud-crab *Rhithropanopeus harrisi* (Christiansen et al., 1977a,b). Methoprene also retarded growth in early larval stages and postlarvae of the estuarine shrimp, *Palaemonetes pugio*, although enhancement of growth was recorded in the premetamorphic stages (McKenney and Celestial, 1993).

The similarity between the results of previous studies and the present study suggest that MF acts in a manner similar to juvenile hormone in insects and may have a physiological role in the regulation of larval development in crustaceans. However, the possibility that these effects represent a case of sublethal toxicity could not be entirely ruled out, e.g. effects of synthetic juvenile hormone analogs in shrimp larvae (McKenny and Celestial, 1993). Moreover, juvenile hormone analogs are commonly used as pesticides, and since *M. rosenbergii* larvae naturally hatch in the estuary they might be exposed to chemicals with juvenoid activity washed from treated agricultural land.

In such cases, comparable effects to those found in the present study could be expected.

This study presents the development and testing of a new method for the administration of MF into crustacean larvae. It may facilitate further studies addressing the question as to the physiological role (Homola and Chang, 1997) and mode of action of MF in the regulation of crustacean larval development and metamorphosis. Such method of administration could be applicable, when needed, in cases of administration of various hydrophobic drugs into prawn and other crustacean larvae.

Acknowledgment

This study was partly supported by a US-Israel ATJV grant. A.S. is the incumbent of the Judith and Murray Shusterman Chair for Career Development in Physiology. The unlabeled and radiolabeled MF were generously contributed by Prof. Hans Laufer, Department of Molecular and Cell Biology, University of Connecticut. We thank Dr. Simy Weil for critical review and Ms. Inez Mureinik for editorial review of the manuscript.

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